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Technical Guide for ELISA

- Protocols
- Troubleshooting

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Who will this help?

The KPL "ELISA Technical Guide" is a continuation of the series of guides and information from KPL to help researchers better understand the available tools for performing improved protein detection experiments. It is designed primarily for the beginner as it explains basic concepts, protocols and troubleshooting of ELISA assays. It will help you to determine which type of ELISA is most likely to give the needed information, how to set up and perform an ELISA and finally how to interpret the data. While the Guide is aimed at beginners, some of the hints, suggestions and troubleshooting should be useful reminders to experienced ELISA mavens.

The Technical Guide is divided into ten sections starting with an overview of the different types of ELISA formats available. This is followed by more specific sections discussing the factors that need to be addressed to develop a successful ELISA: representative protocols, tips that will help improve the precision of an assay; and how to handle and interpret the data generated. Troubleshooting, and Resources sections provide information to help solve the problems encountered in an ELISA.

Help for Beginners:

√ Which Type of ELISA
√ How to Set it Up
√ How to Interpret the Data

Tips for the Experienced

Introduction - What's an ELISA?

ELISA evolved in the late 1960s from RIA (radioimmunoassay) with the observation that either the antibody or the analyte (antigen) could be adsorbed to a solid surface and still participate in specific high affinity binding. The adsorption process facilitated the separation of bound and free analyte, a situation that proved difficult to engineer for many analytes with RIA. Over the intervening years, the term ELISA has been applied to a wide variety of immunoassays, some of which do not employ enzymes and some of which do not require the separation of bound and free analyte. The distinguishing feature of all of these assays remains the use of antibodies to detect an analyte.

ELISAs can be qualitative or quantitative but they all need highly specific and sensitive ANTIBODIES.

In this guide, the discussion of assays is restricted to enzymatic systems that require the separation of bound and free analyte (heterogeneous assays).

ELISA - Why use 'em?

ELISA has become extraordinarily useful because it allows rapid screening or quantitation of a large number of samples for the presence of an analyte or the antibody recognizing it. Variations on this theme are now used to screen protein-protein, protein-nucleic acid, nucleic acid-nucleic acid interactions in microarrays. Other solid supports have evolved such as nitrocellulose and PVDF for blotting (another variation albeit with less ability to quantitate). ELISA, however, remains popular because of its ease of performance and automation, accuracy, and the ready availability of inexpensive reagents.

Some Limitations

One limitation of the ELISA technique is that it provides information on the presence of an analyte but no information on its biochemical properties, such as molecular weight or its spatial distribution in a tissue. To obtain this information one needs to perform other types of assays. For example, blotting assays combine separations based on physical properties of the analyte with detection techniques. Immunohistochemical assays performed on tissue and cells provide information on the specific location of an analyte. (see *Immunohistochemical Staining: Principles and Practices*). Both of these techniques can also provide some quantitation of the analyte, but not as accurately as ELISA.

Antibodies - The Key to an ELISA

The antibody is the major factor determining the sensitivity and specificity of an assay. The structure of antibodies is discussed more thoroughly in "The Use of Antibodies in Immunoassays". Briefly, it is the three dimensional configuration of the antigen-binding site found in the F(ab) portion of the antibody that controls the strength and specificity of the interaction with antigen. The stronger the interaction, the lower the concentration of antigen that can be detected (other factors being equal). A competing factor is the specificity of binding or the cross-reactivity of the antibody to serum proteins other than the the target antigen. Depending on whether the antibodies being used are polyclonal or monoclonal, cross-reactivity will be caused by different forces. In either case driving the assay to the limit of sensitivity may result in cross-reactivity, and one is faced with the conflicting needs of sensitivity versus specificity. We will discuss this further in Section IV.

At first glance, the choices in ELISA formats may be overwhelming, but don't dispair. This chapter will help you make sense of the options. There are a wide variety of ELISA formats available that vary depending on the sensitivity required and whether one is trying to detect an analyte or the antibody response to it. In the following section we will discuss these various configurations and when to use them.

Homogeneous vs. Heterogeneous

Homogeneous ELISA formats do not require separation of reacted from unreacted material in order to detect measure target antigen, usually a hapten. Bound analyte can modify the activity of a labeled detection reagent (e.g. up regulating or down regulating enzyme activity upon binding). In a heterogeneous assay format the bound analyte does not modify the activity of the detection reagent, thus the bound and free must be separated by a washing step after binding in order to distinguish them.

A Basic ELISA

1. Coat solid phase with either antibody or analyte.
2. Block remaining binding sites on the solid phase.
3. Add either analyte or anti-analyte antibody to be detected.
4. Wash out excess reagent. This separates bound from free analyte.
5. If reagent in step 4 is an analyte, add a second anti-analyte antibody with detection molecule attached. If reagent is an anti-analyte antibody, add an anti-Ig antibody with detection molecule attached.
6. Wash out excess reagent.
7. Add substrate. The color change or amount of light emitted is proportional to the level of target analyte.

Capture vs. Direct

Within the heterogeneous type of assay, several different formats can be distinguished based on which component is immobilized. As illustrated in Figure 1, either antibody or antigen (the analyte to be detected) can be immobilized. Antibody immobilized formats are generally referred to as capture or sandwich assays. Either a primary antibody recognizing an epitope of the molecule to be detected, or an anti-Ig (or protein A/G) can be immobilized. This is the preferred format in situations where the antigen is being detected. In contrast, an antigen or epitope can be immobilized and is referred to as a direct assay format. This format is commonly used when the immune response to an antigen is being analyzed.

Assay Formats

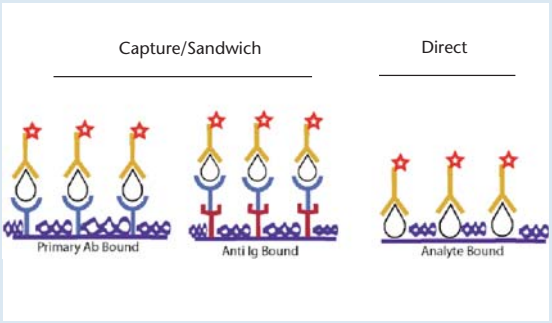


Figure 1.

Competitive vs. Noncompetitive

Each of the above assay types can be adapted to a competitive or noncompetitive format. The distinguishing feature of a competitive assay format is that the combination of an unknown amount of analyte introduced from the sample and the reference analyte compete for binding to a limited number of antibody binding sites. This assay can be performed with either the analyte or the antibody adsorbed to the solid phase. As shown in Figure 2A, added sample analyte is competing

Competitive Assay Format

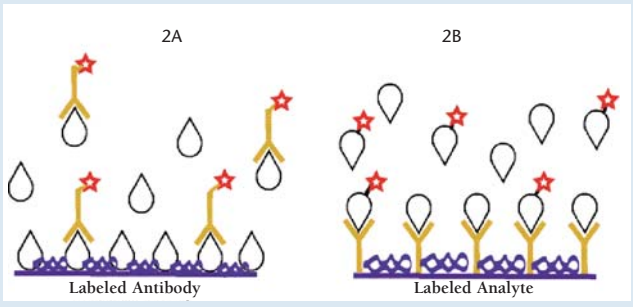
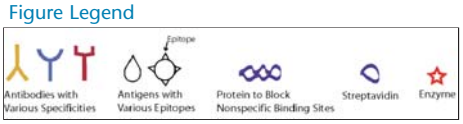


Figure 2.

Figure Legend



with solid phase adsorbed reference analyte for binding to a limited amount of labeled antibody. In Figure 2B labeled reference analyte in solution combined with sample analyte competes for binding to a limited amount of solid phase -- adsorbed antibody.

If a saturating amount of antibody were present, adding a small amount of the sample competitor may not effect a detectable change in activity in the assay. Thus the sensitivity of a competitive assay depends on having slightly fewer antibody binding sites than the number of reference analyte sites. It provides the most accurate quantitation of the different formats available. Because only a limiting amount of antibody can be used, the sensitivity of this format is strictly limited by the affinity of the interaction between the antibody and the analyte. In practice it is not possible to accurately quantitate analyte at a concentration much less than 10 fold below the K (equilibrium constant) of the antibody.

Quantitation can be obtained by generating a "standard curve" of concentration of added competitor analyte vs activity. To do this in the format illustrated in Figure 2A, one would add aliquots of known increasing concentrations of analyte to wells containing the solid phase adsorbed analyte. To each of these wells one would add an aliquot of labeled antibody and generate the curve illustrated in Figure 3. For the format illustrated in 2B, one would mix aliquots of known

Standard Curve in a Competitive ELISA

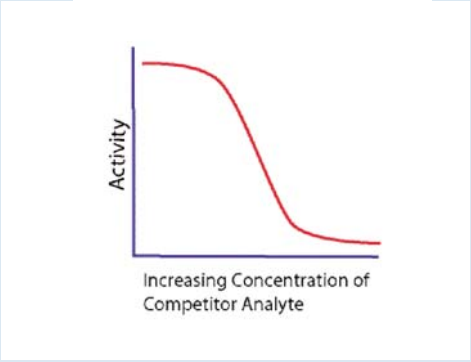


Figure 3.

increasing concentrations of analyte with aliquots of labeled reference analyte and add each mix to a well containing the solid phase adsorbed antibody and generate the curve illustrated in Figure 3. The standard curve should be run each time an unknown is analyzed.

Quantitation in a Competitive ELISA

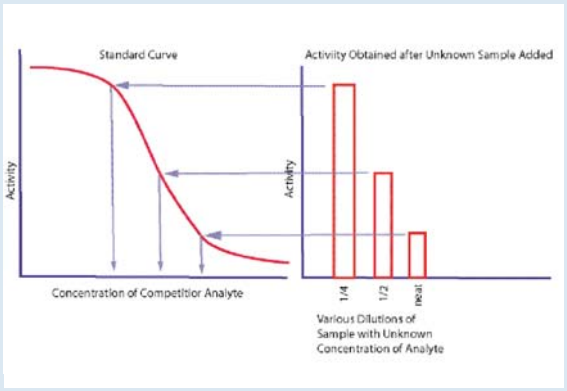


Figure 4.

The sensitivity of a competitive assay depends on having slightly less antibody binding sites than the number of reference analyte sites.

Noncompetitive Format
Highest Sensitivity

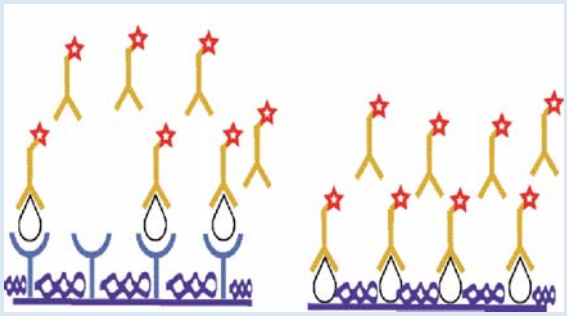
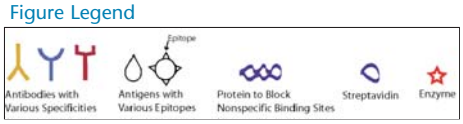


Figure 5.

Figure Legend



Quantitation in a Proportional ELISA

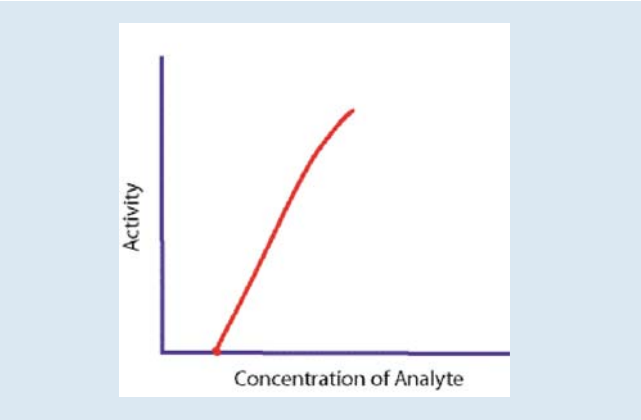


Figure 6.

To quantitate an unknown, compare the activity obtained with the aliquot of unknown to the standard curve as in Figure 4. If the unknown has enough activity, it is advisable to run several dilutions of the unknown. In an ideal situation the activity generated by these dilutions should parallel the dilutions that were made (e.g. a 1/2 dilution should yield 1/2 the activity). If it does not, there are likely to be interfering components in the sample matrix.

In addition, by a careful choice of analyte or epitope, a competitive assay can be made highly specific even in the presence of cross-reactive antibodies.

Noncompetitive ELISA formats are illustrated by the capture and direct assays shown in Figure 5. The distinguishing feature of this format is that antibody-binding sites are present in excess over the analyte being detected. As a result, this format is the most sensitive and can be performed with either the antibody adsorbed to the solid phase or the analyte or epitope adsorbed. Detection limits up to 10^{4} less than the K of the antibody are possible. The first format has been successfully used to quantitate multi epitope molecules (e.g. cytokines) and depends on preparing antibodies to at least two different and non overlapping epitopes, usually monoclonal antibodies. Assays with detection limits of 10^{-15}M have been reported. The amount of signal generated by the binding of the second antibody is proportional to the amount of antigen present and is often referred to as a proportional assay. Quantitation can be obtained by generating a standard curve as illustrated in Figure 6 and comparing the activity obtained with a sample to the activity on the standard curve.

Indirect

The sensitivity of an ELISA can be increased by amplifying the label bound to the detection partner. This is referred to as an indirect ELISA.

In the simplest format, an antibody labeled with one or more detection molecules is bound to the immobilized antigen. In order to increase the sensitivity, the bound antibody is biotinylated in several locations on its surface. It can thus bind multiple streptavidin molecules, each labeled with one or more detection molecules, as illustrated in Figure 7. Alternatively, labeled protein A/G or anti-Ig labeled with detection molecules can be bound to the primary antibody.

Sensitivity Enhancement

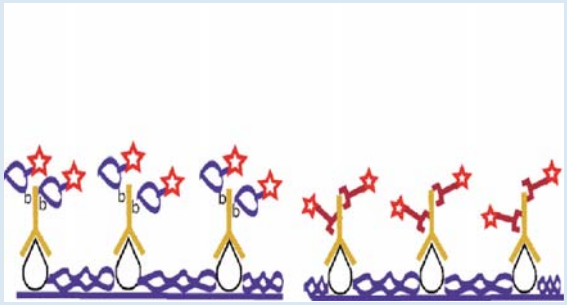
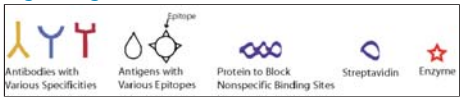


Figure 7.

Figure Legend



What is being detected?

A protein or other large molecule

Now that you know the various formats, let's apply them to what you want to measure. If a protein with multiple epitopes is being detected, a sandwich assay as illustrated in Figure 8 is a good choice. This assay format has been used to both detect and quantitate cytokine molecules. It usually requires two antibodies that react with different epitopes. However, if the molecule has multiple repeating epitopes, it is possible in a sandwich assay to use the same antibody for both capture and detection.

Alternatively, if there is a supply of the analyte to be detected in pure form that can adsorb effectively to a microwell, then one can set up a competitive assay in which the purified analyte is immobilized and analyte in the sample competes with the immobilized analyte for binding to labeled antibody.

Detecting a Protein or an Organism

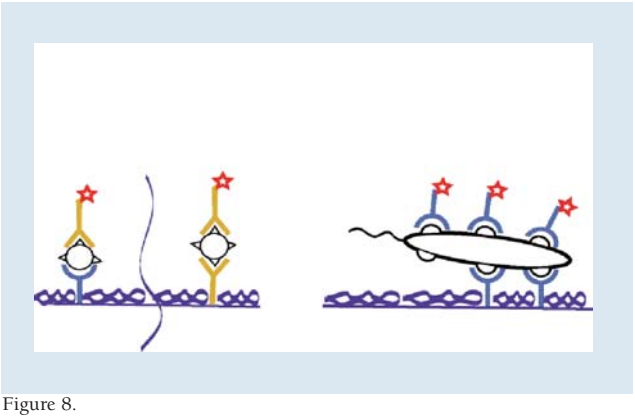


Figure 8.

In this case it is essential to titrate the antibody so that it is limiting, or else the assay sensitivity will be lowered.

An organism

Bacterial or viral assays that detect whole organisms can also use sandwich assays with the same antibody for both capture and detection as illustrated in Figure 8.

A small molecule

If the target molecule is small or consists of a single epitope, a modification of the formats described above is needed. Small molecules by themselves either do not adsorb well to a solid phase, or may be masked by the blocking protein added. However, small molecules can often be attached to larger proteins which provide a means to attach the desired epitope to a solid phase in a configuration that allows the epitope to be bound by an antibody. In order to analyze the immune response to a single epitope, the format illustrated in Figure 9A can be employed. If detecting or quantitating the epitope is desired, typically a competitive format is required, as illustrated in Figure 9B. Another variation, not illustrated, is to add the small molecule as a competitor in format 9A.

Detecting a Small Molecule

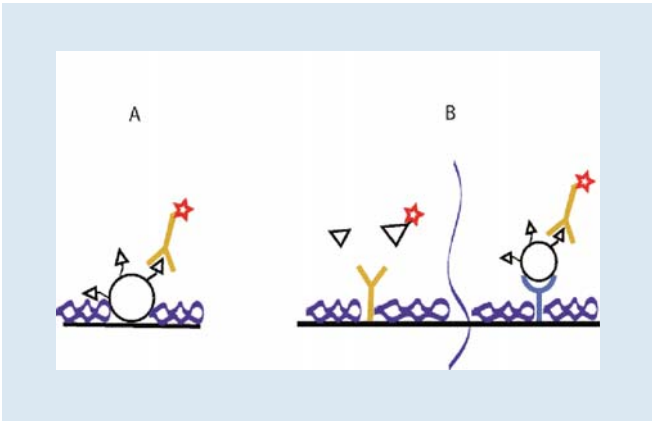
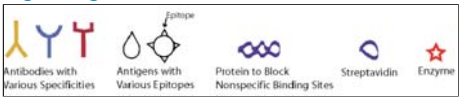


Figure 9.

Is the measurement qualitative or quantitative?

Screening assays where the results are "eyeballed" can easily be performed in noncompetitive formats where a positive result must be discerned over background, especially when the background has been controlled to a non-observable level. On the other hand, in competitive assays a difference in the amount of color is much more difficult to discern by eye. Unless one is looking only for gross differences, it is best to rely on plate readers for quantitation.

Figure Legend



Is the measurement of the antibody response to a molecule?

Antibody responses to an epitope, especially if the epitope is on a large molecule, are typically easy to follow in a direct assay format. Responses to haptens are easily analyzed by attaching the epitope to a large protein that can be adsorbed to the solid phase as illustrated in Figure 9A.

What level of sensitivity is required?

The factors that determine the ultimate sensitivity of a competitive assay are the antibody affinity constant and the experimental errors. The detection limit of the substrate is not typically limiting. It has been calculated theoretically that with a $K = 10^{12} M^{-1}$ (an extraordinarily high equilibrium constant for an antigen-antibody interaction) and a 1% coefficient of variation (CV) for the response at zero dose, the lowest detection limit possible would be 10^{-14} M. A more easily achievable limit would be 10^{-9} - 10^{-10} M.

The factors limiting the sensitivity of a sandwich assay are the affinity of the antibody, the experimental errors and the nonspecific binding of the labeled antibody, expressed as a percentage of the total antibody. It has been estimated that with a $K = 10^{12} M^{-1}$, 1% CV of the response at zero dose, and a 1% nonspecific binding of the labeled antibody, the detection limit can be as low as 10^{-16} M. In addition, this can be enhanced further by using more sensitive detection substrates.

Need more Sensitivity?

To get the most sensitivity from an assay, the following factors must be addressed:

- Background noise, can usually be minimized by optimizing the blocking and washing steps. The lower the signal, the lower the background must be in order to detect a positive result.
- Low signal due to low level attachment of the bound molecule can often be overcome by testing different plates or by switching to covalent linkage to the plate.
- Low signal can be amplified by incorporating indirect labeling techniques or by switching from chromogenic to chemiluminescent substrates.
- Low signal can sometimes be amplified by increasing the incubation times, allowing the binding steps to come to equilibrium.

Which format for me?

Measure	Use
Analyte	Capture or Competitive
Immune Response to an Analyte	Direct or Indirect

It can be a daunting task getting all the factors to mesh to yield an ELISA with high signal-to-noise. Don't despair! ELISA is a robust technique and even a moderate signal to noise can be useful - the key is to make it reproducible. The following section will give you the information you need to start your ELISA. We will also discuss some issues to think about that may help you to further enhance your signal-to-noise.

We will start with some background to help you understand how analytes bind to a plate and what to do if your analyte does not bind. We will discuss five key ELISA factors — coating, blocking, washing, antibodies, and detection molecules. We will look at a method you can use to optimize the concentration of reagents that you will use, some controls to add to insure that your signal really is signal, and finally some representative protocols to start you off.

The Solid Phase

Types of ELISA Plates

Most plates used in ELISA are either polystyrene or derivatives of polystyrene obtained by chemical modification or irradiation of the surface. The most common configuration is 96 wells organized into 8 rows and 12 columns. Each well holds approximately 350 ul of volume with an internal area of approximately 2.5 cm^2 . More recently, 384 well and 1536 well plates have been developed with the same overall dimensions as the traditional 96 well plates. They are used in high throughput screening. In addition, 96 well plates with wells of one half the volume of the traditional wells are available. Assays performed in the half volume wells are identical in performance to the traditional size, but afford a considerable savings in reagents. Another recent innovation has been to configure the wells so that the area where the bottom meets the side of the well is rounded instead of at a 90 degree angle. This has been reported to afford better washing of the well. Yet another alternative has been to add fins to the inside of the well in order to add more surface area for adsorption. Reports have shown a 10% - 20% increase in adsorbed IgG under certain conditions using these "Star Wells".

Polystyrene is a notoriously poor conductor of heat. Insure that the plate and all reagents are at reaction temperature before beginning an ELISA.

In addition to uncoated plates, there are a variety of modifications that leave amine or reactive groups such as maleimide, hydrazine or N-oxysuccinimide groups on the surface that can be used for the covalent linkage of proteins.

Sources of ELISA plates

There are a number of sources of ELISA plates. A partial listing of suppliers are in Chapter IX, Other Resources.

Plate Surface

Forces Holding Proteins on a Plate

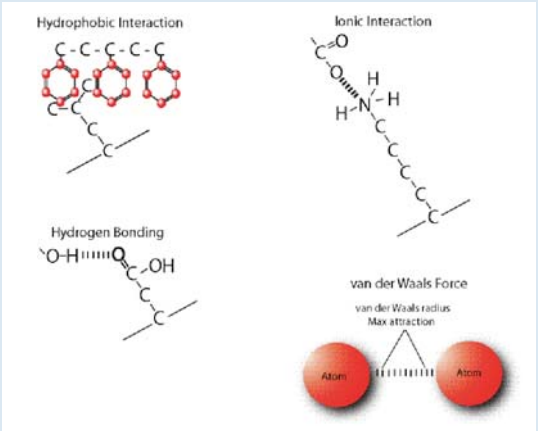


Figure 10.

Polystyrene is composed of an aliphatic carbon chain with pendant benzene rings on every other carbon. This provides a very hydrophobic surface and plates of this type are typically referred to as "medium" to "low binding". In order to enhance binding, manufacturers have modified the surface through techniques such as irradiation which breaks a certain number of the benzene rings yielding carboxyl (COOH) and hydroxyl (OH) groups. The presence of these

groups provides an opportunity for hydrophilic interactions. Plates modified in this way are typically referred to as “high binding”. It has been postulated that the increased binding capacity of treated polystyrene is due to a tendency of IgG molecules to bind to high binding plates preferentially through their Fc region and become oriented "standing up" rather than "lying on their side". In any case, high binding plates do show increased IgG binding to their surfaces when compared to medium binding plates.

The forces that passively adsorb proteins to the surface of high binding plates are hydrophobic interactions, van der Waals forces, hydrogen bonding and ionic interactions in order of increasing strength. They are illustrated in Figure 10. Each bond formed using these forces is 1000 - 100 fold weaker than a covalent bond. In order to have any stability of binding, each molecule must make many weak bonds with the plastic surface. The weakness of these bonds also explains why adsorbed proteins can be leached from the surface of a well. Detergents such as Triton X-100 and Tween-20 are especially effective at blocking hydrophobic interactions between a protein and polystyrene and causing desorption of adsorbed proteins. They should be avoided during the adsorption or blocking steps but may be incorporated at later steps into washing solutions at low concentration.

Which Analytes Bind to Polystyrene

Direct Adsorption to Polystyrene
Proteins
Peptides longer than 15-20 amino acids
Small molecule epitopes attached to a protein
Bacteria and virus

Covalent Linkage
Heavily glycosylated proteins
Proteins in the presence of detergents
Carbohydrates
Short Peptides
Lipids
DNA

Adsorption to a Solid Phase

Polystyrene will bind a wide variety of proteins in an increasing amount depending on their concentration in the coating solution. The specific and optimal amount needs to be determined for each protein, but some general observations have been made for antibodies. Medium to low binding plates bind typically up to 100 - 200 ng of IgG/ cm^2 while high binding plates typically can bind up to 400 - 500 ng of IgG/cm^2. The amount adsorbed has been shown to proportionally increase with the concentration of protein used to coat the well. Thus IgG-coated wells will yield increasing signal as the coating concentration increases.

In addition to proteins, polystyrene plates will adsorb peptides generally of 15 - 20 amino acids in length. In order to achieve strong binding, a peptide will need both hydrophobic and hydrophilic interactions. Typically a drawback to adsorbing peptides directly is that they tend to have few epitopes, and if these are involved in interaction with the plastic, it will be difficult for an antibody to bind to them. One alternative is to attach the peptide to a larger protein through a spacer arm that provides some distance between the peptide and the protein, allowing the antibody to interact with the peptide.

The above strategy can also be used for attaching small molecules to a plate. Using a cross linker one can attach a small molecule to a spacer, leaving some distance between the molecule and the protein.

Bacteria and virus typically have a variety of proteins on their surface that can be directly adsorbed to plastic.

Carbohydrates and heavily glycosylated proteins do not adsorb well to polystyrene by the forces described above because they have very little ability to participate in hydrophobic interactions. In order to adhere these molecules, one must resort to the covalent linkages described below.

If the strategy of attaching peptides to a protein for attach-

ment to the plate is not desirable, they can be readily attached by covalent linkage.

Coating buffers should not contain extraneous protein or detergents.

Membrane proteins released from cells and maintained in solution by detergents are also not adsorbed well in the presence of detergents. Covalent linkage or reduction of the detergent concentration are the best means for attaching these proteins. In fact, covalent linkage can be performed in the presence of detergents such as Tween-20 and Triton X-100.

Edge Well Effects

This phenomenon refers to the observation that occasionally ELISA results show a variance in their ODs between edge wells and the wells in the central region of the plate. In the past this has been attributed to manufacturing variations in plates leading to higher adsorption in the outer wells. More recently manufacturers have begun demonstrating and certifying the consistency of the manufacturing process by testing and reporting the well-to-well variation of replicates performed in all wells of a plate. For instance, Nunc certified plates are guaranteed to have a CV in replicate wells of <5% and no wells with >10% variation.

The continuing observations of edge effects are due more to temperature variations between the middle and the edge of a plate and to differential evaporation from wells. Polystyrene is a notoriously poor conductor of heat; thus, if there is a temperature differential between either the reagents and the plate and/or the plate and the surrounding environment a potential exists for edge effects. If reagents are added while at 4° C, and the incubations are performed at either room temperature or 37° C, a potential exits for uneven temperature equilibration. At different temperatures wells will produce different reaction rates. This potential also exists when plates are stacked on top of each other. The temperature at the outside may be 1 - 2 degrees different than on the inside wells located on plates in the middle of the stack. One way to avoid these problems is to pre-equilibrate all reagents to the incubation temperature before adding to plate wells.

Working with peptides?

If you have adsorbed a peptide but can't detect it -

- attach the peptide to a large protein first and adsorb that to the plate
- covalently link the peptide to the plate

Plates should be sealed during incubations to avoid evaporation from the well, especially if incubations are being performed at 37° C or for extended time periods. In addition, care should be taken to insure that all wells of the plate are effectively sealed in order to avoid uneven evaporation.

Covalent Coupling to the Solid Phase

A number of modifications have been made to the polystyrene surface that allow for covalent linking of molecules to the plastic surface.

Amine

Both Nunc and Corning provide surfaces modified by a short spacer with an amine at the end. Approximately 10^{14} amine sites per cm^2 can be covalently linked to the following substances:

- other small molecules, e.g N-hydroxysuccinimide-biotin
- peptides either through the COOH end by using a cross linker such as carbodiimide or through the amine by using a homobifunctional cross linker such as disuccinimidyl suberate (DSS)
- proteins either through an amine using a homobifunctional cross linker or through a COOH using a heterobifunctional cross linker such as N-Maleimidobutyryloxy succinimide ester (maleimide and NHS)

An approximately 40% enhancement in signal generation has been reported using DSS to covalently link alpha feto protein (AFP) to an amine plate versus noncovalently adsorbing to a high binding plate.

Maleimide, Hydrazine and N-oxysuccinimide

Maleimde groups react with a sulfhydryl forming a covalent link between the plastic surface and a protein or peptide. Hydrazine reacts with aldehydes generated by periodate oxidation of carbohydrates. N-oxysuccinimide reacts with amines on peptides or proteins.

Typically proteins do not have free sulfhydryl groups: they are usually cross linked to form disulfides. In order to generate a free sulfhydryl one must either reduce some or all of the protein's disulfides and risk denaturation, or generate a free sulfhydryl by reacting a free amine with the cyclic thioimidate, 2-iminothiolane. This reaction will leave a short spacer with a free sulfhydryl that can react with a maleimide.

The carbohydrates on a protein can be oxidized with $NaIO_4$ to generate aldehydes. The aldehydes can then react with either amines or with hydrazine that are covalently attached to the plastic surface. The Schiff bases and the hydrazones that are formed respectively as a result of the reaction need to be reduced to a more stable linkage by reduction with Na cyanoborohydride. The resulting covalently linked proteins are quite stable.

N-oxysuccinimide (NOS) directly reacts with primary amines found on proteins and peptides optimally at pH 8 - 9. As NOS reacts with any amine, buffers should not be Tris-based. In addition, the NOS group is easily hydrolyzed, and one should keep the protein concentration as high as practical.

Once the reaction to covalently link a molecule to the plastic is completed, one must be very careful to quench any unreacted groups on the plastic with an unreactive peptide or protein and to block the plate with blocking reagents described above. An advantage of covalent linkage is that detergents can be used in washing without fear of releasing adsorbed protein. In fact the initial coupling can be performed in detergents, thus allowing membrane-bound and other insoluble proteins to be coupled. It is advisable to include detergent and/or protein in washes for any plate with a covalently-linked target.

Ideal blocking agents have the following characteristics:

- Effectively block nonspecific binding of assay reactants to the surface of the well
- Do not disrupt the binding of assay components that have been adsorbed to the well.
- Act as a stabilizer (prevent denaturation) of assay reactants on a solid surface
- Are not cross-reactive with other assay reactants
- Possess no enzymatic activity that might contribute to signal generation of the substrate or degradation of the reactants
- Perform all of the above reproducibly from lot-to-lot

Initial Steps in Developing an Assay

Coating

Buffer

Due to the predominantly hydrophobic nature of polystyrene surfaces, adsorption will best take place at a pH at or slightly above the pI (pH at which the + and - charges are balanced and there is no net charge on the protein) of the protein being adsorbed in order to avoid electrostatic repulsion. In the case of antibodies, adsorption is optimal at pH 7- 9 in a salt concentration that helps maintain solubility and native conformation of the protein. Three widely used coating buffers for antibodies are 50mM carbonate pH 9.6, 10mM Tris pH 8.5 and 10mM PBS pH 7.2. However, if a different protein is being coated, one should test a variety of pHs to insure optimal coating. Coating buffers should not contain extraneous protein or detergents. Extra protein is added in the blocking step to block all unoccupied sites on the polystyrene. Detergents should definitely be avoided as a diluent during the coating process. They effectively bind to

The most widely used coating buffers are:
50mM Carbonate pH 9.6
10mM Tris pH 8.5
"10mM PBS pH 7.2"

The most typical protein blocking agents are:

- "Bovine serum albumin - BSA"
- "Non-fat dry milk - NFDM"
- "Normal serum"
- "Casein or caseinate"
- "Fish gelatin"

hydrophobic areas of the protein and the polystyrene and prevent binding. Once binding has occurred, detergents at low concentration can be effective components of washing buffers since they strongly inhibit protein interactions and dissociate those that are weak (typically the nonspecific interactions).

Time and Temperature

Time and temperature are the **most important factors controlling the amount of protein adsorbed**. The most thorough adsorption and lowest well to well variation occurs overnight (16 - 18 hours) at 4° C with the wells sealed to prevent evaporation. Adsorption time can be speeded up by incubation at room temperature (4 - 8 hours) or 37° C (4 hours). However, since polystyrene is a notoriously poor conductor of heat, this shortcut is not advisable unless care is taken to insure even temperature across the plate.

Purity

Another factor influencing the activity of the coated component is its purity. The component to be coated should be highly purified, preferably to homogeneity. Otherwise sensitivity is reduced and the potential for cross-reactivity is increased. When coating antibodies it is important to remember that an antiserum contains at best 5 - 10% specific antibody, the remaining protein consisting of antibodies to other pathogens resulting from exposure of the host and approximately 50% albumin.

Blocking

Coating of wells with the specific binding partner, either antigen or antibody, leaves unoccupied hydrophobic sites on the plastic. These sites **must** be blocked in order to prevent non specific binding of subsequent reactants. If this is not effectively accomplished, the assay will suffer from high background signal and lowered specificity and sensitivity. Blocking reagents are typically chosen in an empirical manner. The optimum blocker for one assay may not perform well in other assays. The two major classes of blocking agents that have been tested are proteins and detergents.

Detergents come in three classes: non ionic, ionic and zwitterionic. Ionic and zwitterionic detergents are poor blockers and should not be used. Nonionic detergents such as Tween-20 and Triton X-100 have been used with reagents covalently linked to the solid surface, but are best used as washing agents where they can disrupt undesired protein-protein interactions. Their main disadvantages as blocking agents are:

- They disrupt the hydrophobic interactions that bind proteins coated to the surface of the plastic.
- They block only hydrophobic sites and leave sites that can participate in hydrophilic interactions unblocked.
- They can be washed away with aqueous washing buffers when they contain a detergent at a concentration above the critical micelle concentration.
- They can interfere with enzyme activity and thus reduce signal generation.

Proteins on the other hand can block both the unoccupied hydrophobic and hydrophilic sites on the surface of the plastic and can serve as stabilizing agents, thereby preventing denaturation as proteins react at the surface in a solid phase assay.

It may be sufficient in some assays to add the protein blocker at the blocking step and leave it out of the washing buffers. However, if high background persists, adding protein to the wash solution may lower the background.

Bovine serum albumin is the most common blocking agent. It is typically used at a concentration between 1% and 5% in PBS at pH 7. It is inexpensive and can be stored dry at 4° C degrees or at 10% to 20% concentrates. Special grades of BSA that are DNase- and RNase-free; fatty acid-free; and/or ELISA-tested may be obtained. They typically have low lot-to-lot variability. More crude preparations such as "fraction V" may contain phosphotyrosine and should be avoided as it may cross-react in the assay. If BSA is used as the carrier protein in eliciting anti-hapten antibodies, it should be avoided in the blocking step.

Non-fat dry milk (NFDM) is typically used at a concentration of 0.1% to 0.5%. The main drawbacks are lot-to-lot variability in preparations and a tendency to cause lower signal. NFDM is also prone to varying concentrations of biotin which may interfere when using streptavidin - biotin linkages to attach a streptavidin-enzyme conjugate to a biotinylated antibody. NFDM is also prone to deteriorate rapidly and should be made up fresh every 1-2 days. Some lots of NFDM have also been shown to inhibit alkaline phosphatase activity.

Casein or caseinate. Caseinate is the more soluble version of casein (partially digested by NaOH). Casein is the main protein component of NFDM and may be a better choice for a blocking agent because it lacks most or all (depending on the grade) of the impurities found in NFDM.. The caseinate version has been shown in some cases to be a better blocker than BSA, possibly due to its smaller size. It is generally used at concentrations of 1% to 5%. If sensitivity is an issue, it is worthwhile to test caseinate as a blocker either alone or in combination with BSA.

Normal serum has been used at concentrations of 5% to 10%. It is also a very good blocking agent due to the diversity of proteins within the mixture, although albumin will be the most prevalent protein. A definite disadvantage is the presence of Ig within the mixture. Most anti-mammalian Igs cross-react with each other. One should carefully consider whether this potential cross-reactivity will be an issue before using normal serum. As an alternative, chicken Ig and fish Ig have very little cross-reactivity with anti-mammalian Ig and should be considered.

Fish gelatin has been used between 1% to 5% as a blocking agent. This agent works very well as a blocker in Western, Southern or Northern blotting applications, but not nearly as well in ELISA applications. Gelatin is more efficient at reducing protein-protein interactions than in blocking hydrophobic sites on plastic, which may make it more useful in washing buffers to reduce nonspecific interactions. Fish gelatin is a good choice as it does not gel at room temperature as do the gelatins of most mammalian species. Moreover, cross-reactivity is limited.

Storage of Coated Plates

If coated plates are going to be used immediately there is no need for further processing. Keep the wells in blocking solution until the first reactant is ready to be added. If plates with adsorbed antibody are allowed to dry for as short as 20 minutes without further processing, partial loss of activity is possible.

If it is desired to store the plates before using them, remove all the liquid at the end of the blocking step, fill the well with 2% sucrose and incubate for 5 - 10 minutes. Remove all the liquid and allow to dry for 1 - 2 hours at room temperature. Store in a sealed plastic bag with desiccant. It is also recommended to dilute the blocking buffer and sucrose in PBS. Phosphate has the ability to structure water molecules around the surface-bound molecules while other buffers such as carbonate do not.

It is highly recommended that testing of these drying conditions be performed on the particular molecules being adsorbed before full scale processing begins. Coat, dry and store a set of plates for at least 1 - 2 days and compare the activity to freshly made plates using the same batch of coating reagent.

Washing

The incubations that are performed in an ELISA allow high affinity specific interactions to form among reactants. By washing several times between each incubation, the excess reactants are diluted to an undetectable background level. In addition to the specific high affinity interactions, there are

always low affinity interactions that occur between the reactants. The wash buffer must be able to disrupt low affinity interactions to allow these reactants to be washed away.

The wash solution should consist of a physiological buffer to avoid denaturation of the two binding partners and to preserve enzyme activity. Buffers such as PBS, Tris saline or imidizole-buffered saline at neutral pH are ideal for this. Imidizole is a very favorable buffer. It is compatible with all enzyme systems and has been reported to increase the activity of HRP. Sodium azide should be avoided when using HRP as well as phosphate buffers when using alkaline phosphatase as the reporting enzyme.

When using alkaline phosphatase a final wash in a buffer at pH 9-10 may enhance enzyme activity.

In order to disrupt low affinity nonspecific interactions a detergent should be included in the wash buffer. Tween-20 or Triton X-100 work well for this purpose at concentrations of 0.01% to 0.05%. A final wash step using a buffer without detergent may be advisable if detergent will affect the activity of the enzyme.

If high background persists, adding protein to the wash solution may lower the background.

In order to effectively dilute the excess reactants, it is necessary to wash 3 - 5 times after each incubation. It is also a good idea to allow a 5 - 10 minute soak with wash buffer at each wash step. This will allow the disruption of low affinity nonspecific interactions to come to equilibrium. If the wash steps are being performed by hand, tap out the excess wash buffer at each step by banging the plate upside down on dry paper towels. Do not allow the plate to dry for extended periods between wash steps as this can lead to a reduction of activity.

Antibodies

See "The Use of Antibodies in Immunoassays" for a description of the structure and function of antibody molecules.

Antibodies are the key to an ELISA and provide the basis for its specificity and sensitivity. However, these two factors are often competing. An antiserum, made up of a mixture of antibodies from many different B cells, is referred to as polyclonal. Each antibody molecule within the mix has a high degree of specificity for a single epitope, but the mix has reactivity to many epitopes. In most antisera there are, in fact, reactivities to epitopes that were not even part of the planned immunization, but are due to the animal's response to pathogens it has seen over its lifetime. Over the course of a planned immunization, antibodies reactive to the epitopes that are injected become dominant and others become a minor part of the antiserum. However, as sensitivity is pushed to its limit, the reactivities of these minor components may become visible. There are two ways to circumvent this issue: purification of the antiserum or design of the assay. For a description of affinity purification of antiserum see How KPL Purifies its Antibodies . The second method requires careful design of the assay. In setting up a capture assay where one of the antibodies is polyclonal, the other should be a monoclonal. In a competitive assay, specificity can be engineered by careful design of the competitor antibody. If sensitivity is not an overriding concern, it may be possible to dilute out the cross-reactivities and not see them.

Another source of cross-reactivity lies in the specificity of each antibody molecule. The strength of the interaction between the antibody binding site and the epitope lies in part in the complementarity of their three dimensional structure. It is easy to imagine two different large protein molecules, each having many epitopes with similar though not identical three dimensional shapes. In fact it is also not hard to imagine these two proteins sharing an identical epitope. This type of cross-reactivity is shared by both polyclonal and monoclonal antibodies.

Coating the Plate with Antibodies

How much

As mentioned before, medium to low binding plates bind typically up to 100 - 200 ng of IgG/ cm^2 while high binding plates typically can bind up to 400 - 500 ng of IgG/cm^2. The amount of antibody adsorbed has been shown to proportionally increase with the concentration of protein used to coat the well. Thus IgG coated on wells generates increasing signal as the concentration of coating goes from 0.1 ug/ml to 10 ug/ml. As the amount of antibody bound reaches saturation, it appears to form a monolayer on the surface of the plastic. It has been observed that further increasing the amount of Ig added leads to an unstable condition in which sensitivity actually begins to decrease. It has been postulated that this is due to the formation of a multi layer of Igs in which some Igs bind to other Igs through protein-protein interactions instead of directly to the plastic. Analyte that binds to an Ig that is not securely bound to the plastic is thought to be removed during washing steps, leading to the appearance of decreased sensitivity as illustrated in Figure 11.

Partial denaturation

Hydrophobic interactions are promoted by exposing the hydrophobic areas of the protein to be adsorbed. It has been observed that increased activity will occur when IgG that has been partially denatured is used for coating. However, this must be done with care so as not to over denature and cause aggregation and precipitation of the protein. It has been postulated that the increased activity is due to the Fc being preferentially denatured and exposing hydrophobic areas while the more stable Fab remains in its native conformation. Partial denaturation has been obtained by exposure to 50mM glycine-HCl pH 2.5 for 10-20 minutes at room temperature or in neutral buffer by adding an equal volume of 6 M urea and incubating overnight. These steps should be followed by dialysis into the coating buffer.

"Hook Effect" in an ELISA as a Result of Excess Coating Antibody

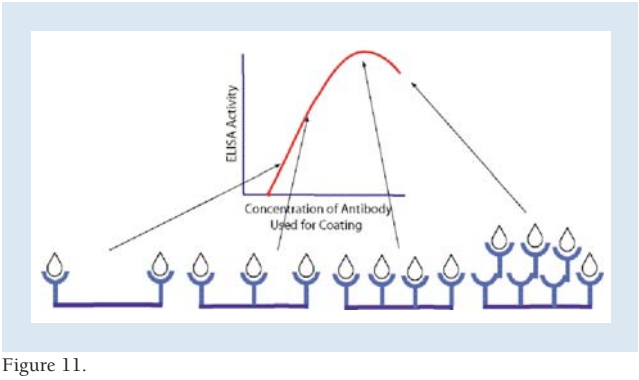


Figure 11.

Purity of Antibodies

In any given antiserum the amount of specific antibody is generally in the range of 5 - 10% of the total immunoglobulin. Antibodies used either as the capture or the detection agent thus need to be purified before use. Antibodies are supplied as polyclonal antiserum or as monoclonal antibodies in ascities or tissue culture media (unless you buy them purified). The following methods can be used to purify both forms.

Antiserum

- Caprylic acid can be used to precipitate most serum proteins, including albumin, leaving the IgG in solution. This can be followed by $(NH_4)_2SO_4$, which precipitates the IgG. The resulting fraction is highly purified and should contain less than 1% albumin.

- Protein A is derived from the cell wall of certain strains of *Staphylococcus aureus*. Protein G is derived from the cell wall of group G strains of *Streptococci*. Both have affinity for IgG Fc regions and can be used to purify IgG. Protein G has a higher affinity binding and a broader species specificity than Protein A. See - "Purification of IgG with Protein A or G Agarose"

- Affinity purification is a good way to increase the fraction of specific antibody in the pool of antiserum. Typically an antigen is attached to a chromatographic matrix such as agarose and the antiserum is passed over the matrix. Antibodies within the mixture that bind to the antigen are retained on the column while nonspecific antibodies are

Antibody Purification

Caprylic Acid Precipitaion

Dilute the serum 1/1 or 1/2 with 60-120mM $Na(CH_3COO)$ buffer pH 4.0

Adjust to pH 4.8

Stir

Dropwise add caprylic acid. Continue stirring for 30 minutes at room temperture

Centrifuge at 5000Xg for 10 minutes

Supernatant contains the IgG

Dialyze against PBS

Amount of caprylic acid per 10 ml of serum
0.4 ml - mouse; 0.7 ml - all others

$(NH_4)_2SO_4$ Precipitation

Determine volume of serum

While stirring add an equal volume of saturated $(NH_4)_2SO_4$

Hold at 4° C overnight

Centrifuge at 3000Xg for 30 minutes

Pellet contains the IgG

Resuspend in 0.1 to 0.5 of the starting volume in PBS

Dialyze against PBS

washed away. This is followed by releasing and recovering the specific antibody. In addition, this is a good technique to increase the specificity of the antibodies by careful choice of the protein attached to the agarose matrix. If the antiserum was raised to a small molecule (hapten), it was attached to a carrier molecule for the immunization. One can at this point attach the small molecule to a different and non cross-reactive protein for attachment to the agarose and exclude reactivity to the carrier. Alternatively, if the immunogen was a large multiepitopic molecule, one can attach antigen to the agarose matrix that carries one or a few epitopes of the immunogen. These are strategies for positive selection.

Negative selection can also be employed. If there are known interfering cross-reactions, employ a negative purification by passing the antiserum over agarose beads bearing the cross-reacting molecules. The unwanted antibodies will bind to the column and the unbound fraction containing the desired reactivity will pass through for collection.

Affinity purification is a powerful tool to control the specificity of the antibodies being used in an ELISA. Affinity purification can also increase the sensitivity that can be achieved by increasing the fraction of specific antibody and thus the fraction of positive antibody that will be adsorbed to a surface. For a further description of this technique see - "How KPL Affinity Purifies its Antibodies".

Monoclonal Antibody

Several of the techniques used for purification of polyclonal antibodies can also be used for monoclonal antibodies. If the antibody is derived from tissue culture, Protein A or G is typically sufficient for purification. If the antibodies are derived from ascities, they will be mixed with other serum components. In this case $(NH_4)_2SO_4$, Protein A or G and affinity chromatography are all useful.

Cross-Reactions

Antibody Cross Reactions

Antibodies, either capture or detection, that are cross-reactive with an epitope shared by the desired antigen and an irrelevant antigen are not uncommon. The epitopes shared by the two antigens may be identical or similar. If the epitopes are similar, there is a good chance that the cross-reactivity with the irrelevant antigen will be lower than with the desired epitope. In this case a longer incubation time allowing the reactants to come to a true equilibrium may help reduce the signal caused by the cross reaction. Alternatively, the presence of a higher salt concentration or the addition of detergents to the reaction mixture may help reduce the low affinity interaction. If the antibody is polyclonal (e.g. goat or rabbit serum) and the cross-reaction is truly problematic, one can attempt to remove the reactivity by affinity purification through attachment of the cross-reacting antigen to an agarose bead. The antibody serum is passed across the beads to remove the unwanted cross-reactivity with the common epitope.

Interference Caused by Heterophile Antibody or Rheumatoid Factor

Masking of Interference by Inclusion of Nonspecific Antibody

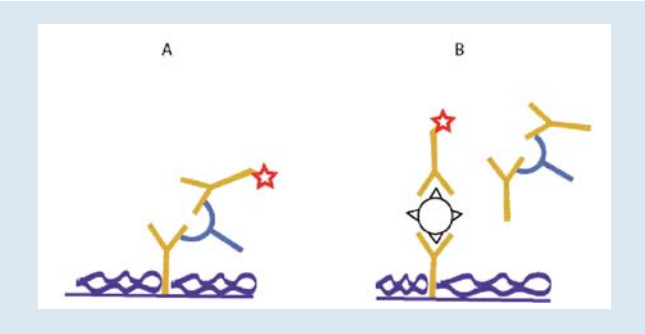


Figure 12.

Another potential cause of antibody interference occurs when the matrix is serum containing heterophile antibodies or rheumatoid factors (RF). These are antibodies in serum that react with IgG, typically the Fc portion, from the individual's own antibody, as illustrated in Figure 12. They are derived from either the individuals previous exposure to pathogens bearing Ig like structures, exposure to Ig from therapeutic treatment, or a genetic predisposition to autoreactivity. These antibodies can lead to a positive result in the absence of true analyte.

One method for reducing interference due to the presence of anti-Ig antibodies is to include nonspecific Ig from the species used to generate the antibodies. A second approach is to use $F(ab')_2$ fragments of the antibodies to prevent binding via the Fc portion. If blocking reactivity or using $F(ab')_2$ fragments is not attractive, interference due to RF can be reduced by physically removal. RF can be precipitated from the sample by adding polyethylene glycol (PEG) or $(NH_4)_2SO_4$ to precipitate Ig.

Monoclonal Antibody Cross Reactions

There is a common misconception that monoclonal antibodies are absolutely specific. This is not true. They are a population of antibodies wth only one binding site. They are specific for a single epitope but may cross-react with epitopes having a similar three dimensional structure with a lower (or higher) affinity. They will also react with two different mulitepitopic antigens if they share the particular epitope recognized by the monoclonal antibody.

Reducing cross reactions with more specific antibodies

Immunogen

If the antigen to be detected is a small molecule (a hapten), the choice of the carrier is very important. The antiserum produced will have antibodies reactive with the carrier as well as the hapten. When setting up an ELISA to detect the hapten, it is critical to attach the hapten to a new carrier (for attachment to the plastic) that is not cross reactive with the carrier used in the immunization.

If the antigen is a large molecule it should be purified to reduce the generation of irrelevant antibodies in the immunogen. The more heterogeneous, the more reactivities the antiserum will have. In addition, the unwanted antigen may be more immunogenic than the desired antigen. If that is the case, the antiserum will be dominated by antibodies to the wrong antigen.

Affinity purified antibodies

See above

Monoclonal Antibodies

Monoclonal antibodies are specific for a single epitope and can be obtained in very pure form. In sandwich assays one can use two different monoclonal antibodies (one for capture and one for detection) reactive with different epitopes. This approach increases the specificity of the assay, since few potentially cross reacting molecules will share two epitopes.

Diluent for antibodies

If the coating antigen was derived from a tissue culture sample or from serum and not purified to homogeneity, there may be proteins adsorbed to the surface that will cross-react with the detection antibodies. In order to mask this reactivity, one can dilute the antibody in tissue culture media or serum.

Comparison of KPL's ELISA Substrates

Substrate	*p*NPP	BluePhos/BCIP	ABTS	SureBlue/TMB	SureBlue Reserve	Luminol/LumiGLO
Enzyme	AP	AP	HRP	HRP	HRP	HRP
Type	Chromogenic	Chromogenic	Chromogenic	Chromogenic	Chromogenic	Chemiluminescent
Detection Limit	10^{-13} moles of AP	10^{-13} moles of AP	10^{-13} moles of HRP	10^{-15} moles of HRP	5 x 10^{-16} moles of HRP	10^{-18} moles of HRP
Kinetics	Fast	Fast	Slow	Fast	Fast	Fast-max light output in 5 minutes
Color Before Stopping	Yellow	Blue	Blue-Green	Blue	Blue	NA
Absorbance Before Stopping	405-410 nm	595-650 nm	405-410 nm	650 nm	650 nm	425 nm (emission)
Stop Reagent	5% EDTA	BluePhos Stop Solution	ABTS Stop Solution	TMB Stop Solution	TMB Stop Solution	NA
Color After Stopping	Yellow	Blue-Purple	Blue-Green	Yellow	Yellow	NA
Absorbance After Stopping	405-410 nm	595-650 nm	405-410 nm	450 nm	450 nm	425 nm (emission)
Amplification After Stopping	None	None	None	2-3 fold	2-3 fold	NA

Detection Molecules

In the mid-1960s immunoassays were developed using radiolabels as the detection molecule (RIA). Since then other detection molecules have been developed. The most widely used are enzymes that can convert a colorless substrate to a color or convert a non-luminescent molecule to a luminescent one. The assay is quantitative since the amount of color generated is proportional to the amount of enzyme present. Alternatively, fluorophores can be directly attached to antibodies or streptavidin and used as detection molecules. However, they suffer from low sensitivity due to inefficient design of fluorescent plate readers. This problem may be alleviated by fluorophores with large Stokes shifts and better design of instruments.

Typical enzymes used in immunoassays are listed in Table 1. The most widely used are horseradish peroxidase and alkaline phosphatase.

Horseradish peroxidase

HRP and Conjugates

Horseradish peroxidase (HRP) is a holoenzyme with hemin as the prosthetic group. Removal of the Fe from HRP by EDTA will inactivate the enzyme. The molecular weight is approxi-

Enzymes for ELISA

Enzyme	Source	pH Optimum	MW	Sp Act	Substrates
Alkaline Phosphatase	calf intestine	9-10	100,000	1,000	*p*NPP abs max = 405nm BluePhos abs max = 600nm
beta-galactosidase	*e.coli*	6-8	540,000	600	oNPG abs max = 420 nm
peroxidase	horseradish	5-7	40,000	4,500	ABTS abs max = 415 nm TMB abs max = 450 nm

Table 1.

mately 40,000 daltons with 8 carbohydrate side chains that are typically used to conjugate HRP to antibody or streptavidin. When lyophilized and stored at 4° C, either as the native enzyme or as an antibody/streptavidin conjugate, HRP is stable for several years. In solution HRP and its conjugates are stable for up to a year at 1.0 mg/ml at 4° C in a suitable buffer. Dilute solutions, in the range of 0.1 mg/ml, are not stable and lose significant activity in weeks. This loss of activity at low concentrations can be minimized by stabilizing buffers. In addition, HRP is inactivated in the presence of >100mM phosphate buffers. This inactivation can be minimized by using citrate or phosphate buffers at 20mM. It has also been reported that HRP and its conjugates are inactivated by exposure to polystyrene. This can be prevented by the addition of Tween 20 to the buffer and points out the importance of blocking the plate with protein (see Blocking section).

The reaction scheme of peroxidase involves the oxidation of the enzyme by H_2O_2 to form an intermediate referred to as HRP I. HRP I is in turn reduced by a hydrogen donor via a one electron transfer to form another intermediate (HRP II) and a donor radical. HRP II is further reduced by an additional hydrogen donor via a one electron transfer to regenerate the original enzyme and another donor radical. The two donor radicals combine to yield a detectable product. In the presence of excess H_2O_2 additional intermediates (HRP III and IV) may be generated which lead to inactive peroxidase. The optimal pH is approximately 5.0. The advantage of HRP is its high rate constant which generates signal quickly. The disadvantage is that HRP is inactivated by excess substrate which leads to loss of signal generation at later time points. The consequence of this is less significant when using chromogenic substrates, as once signal is generated it continues. However, chemiluminescent substrates emit light that diminishes after 1 - 2 hours. Results are less sensitive than if read at 5 - 10 minutes after adding substrate.

HRP Substrates

A variety of aromatic phenols or amines can serve as the hydrogen donor. 2,2-azino-di (3-ethyl-benzathiazoline) sulphonic acid (ABTS) and 3,5,3',5'- tetramethylbenzidine (TMB) are the two most popular chromogenic substrates. ABTS yields a blue-green soluble colored product with an absorbance maximum at 405 nm. ABTS has a slower turnover rate than TMB and its reaction rate is significantly slower. However, the dynamic range is very broad, and it is a good choice if sensitivity is not an issue. TMB yields a blue colored product with an absorbance at 650 nm. If TMB is acidified to stop the reaction, it turns yellow with an absorbance maximum at 450 nm and a 2 -3 fold increase in sensitivity. Typically TMB has a detection limit 10 - 50 times lower than ABTS. While the detection limit is more a function of the antibodies being used, TMB can easily detect in the range of 0.1 - 0.3 ng/ml of HRP-IgG.
Less popular chromogenic substrates for HRP are o-

phenylenediamine (OPD), o-dianisidine (ODIA) and 5-aminosalicylic acid (5AS).

HRP chemiluminescent substrates are typically based either on luminol or acridinium esters. As was the case for the chromogenic substrates, HRP is first oxidized by H_2O_2. Luminol, a cyclic diacylhydrazide, is then oxidized by HRP to a radical which forms an endoperoxide which spontaneously decomposes to a dianion that emits light as it returns to its ground state. This process can be enhanced by phenolic compounds that intensify both the light emission and duration of the signal. The light emission has a maximum at 425 nm which can be captured by photodiode or photomultiplier based luminometers.

Alkaline Phosphatase

AP and Conjugates

Alkaline phosphatases are zinc metaloenzymes with 2 atoms of zinc per molecule. The molecular weight is approximately 100,000 daltons depending on the source of the enzyme. Zinc is found in the active site and is required for activity. Removal will inactivate the enzyme. AP catalyzes the hydrolysis of an orthophosphoric monoester to yield an alcohol and an orthophosphate. Optimal catalysis occurs at approximately pH 9. Typical buffers for use with AP conjugates are Tris; borate and carbonate. One should note that the high concentration of Pi in phosphate buffers acts as an inhibitor of AP and should be avoided when using AP as the enzyme. As AP has a lower rate constant than HRP, turnover of substrate occurs more slowly. However, there is no substrate inhibition of AP and the reaction can continue for days. This is most easily seen in the case of the chemiluminescent substrates.

AP Substrates

The typical substrates for AP are *p*-nitrophenyl phosphate (*p*NPP) which yields a soluble yellow colored product with an absorption maximum at 450 nm. Most liquid preparations of *p*NPP are slightly unstable and will turn yellow with storage at 4° C. It can reach an absorbance of ~ 0.1 - 0.2 within 1 - 2 months. To avoid this problem the substrate can be stored frozen. Another alternative is to use *p*NPP in tablet form and dissolve a tablet in buffer just before use. A sensitive alternative substrate is a formulation of Bromo-chloro-indoxyl phosphate (BCIP) that has been rendered soluble. BCIP is usually a precipitating substrate for use on membranes, but this formulation will remain soluble, with the additional advantage that it is very stable and will not undergo an increase in background with storage at 4° C. It is approximately 2 fold more sensitive than *p*NPP.

Conjugating Detection Molecules to Igs and Streptavidin

In order to be used in an ELISA the detection molecule must first be attached to an Ig or Streptavidin (SA) with minimal disruption of the activity of either one. Anti-Igs of many varieties conjugated with either HRP, AP, beta-galactosidase or biotin are available as is streptavidin conjugated with HRP or AP. Several methods are available to conjugate a primary antibody or an analyte. Either an amine, a sulfhydryl or an aldehyde is needed to accomplish the conjugation. The most popular is an amine which can react with gluteraldehyde, the aldehydes created by periodate reaction or the N-hydroxysuccinimide groups on heterobifunctional cross linkers.

It is beyond the scope of this manual to detail the methods for conjugating antibodies or analytes to detection molecules, but several books and articles listed in Other Resources go into detail on this topic.

Optimizing the Reagents

Capture Assay Format

Which antibody to adsorb and which to use in solution?

In a capture or sandwich ELISA format, two antibodies with different specificities are required. Often both are monoclonal. One antibody is the capture and is adsorbed to the plate, and the other is the detection and is in solution. There can be a dramatic difference in the sensitivity of the assay depending on which antibody is adsorbed and which is used in solution. Typically the antibody with the higher

Starting Ab Concentration - Capture	
Coating	1 - 15 µg/ml
Detection	500 ng/ml - 10 ng/ml

affinity should be adsorbed to the plastic. The results of switching capture and detection is illustrated in Figure 13.

If a capture assay is planned, the optimal amount of antibody bound to a plate should be determined. This can be accomplished during the optimization phase by titrating added Ig from 1µg/ml up to 15-20µg/ml. The latter concentration should be sufficient to demonstrate saturation.

In this format both the capture and detection antibody need to be optimized at several (at least three) concentrations of target. This can be done on a single plate since a great deal of information already exists on coating antibodies onto plastic. Divide the plate into four quadrants as illustrated in Figure 14. Each quadrant will receive a different concentration of detection antibody. Coating antibody can be tested at three concentrations between 1µg/ml and 15µg/ml.

Quadrant I - The first two columns (1 & 2) test the low concentration of capture antibody. Columns 3 & 4 test the mid range concentration of capture antibody, and columns 5 & 6

Assay Dependence on Affinity of Solid Phase Antibody

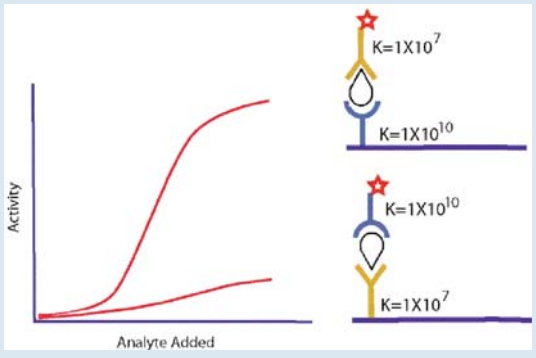


Figure 13.

Optimizing a Capture Assay

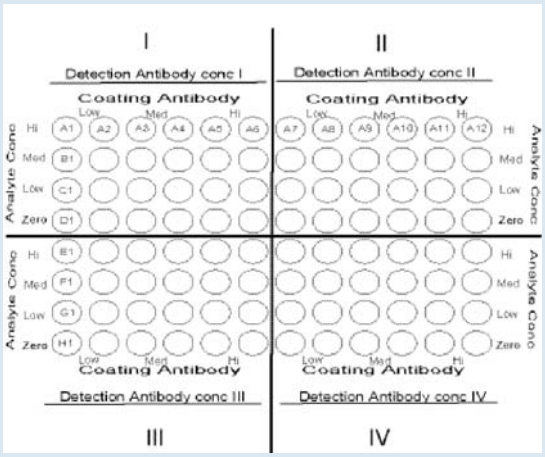


Figure 14.

test the high range concentration. Row A contains target at a concentration in the high range. Row B target in the mid range and row C target in the low range. Row D is a blank receiving no target. All wells of quadrant I receive detection antibody at a high concentration (500 ng/ml is a good starting point).

Quadrants II, III, and IV - Repeat of Quadrant I but at different concentrations of detection antibody. The objective is to determine the coating concentration generating the best signal-to-noise ratio while maintaining low background in the blank rows.

Coating buffer pH and times are not likely to need much optimization since a large body of experience exists on coating antibodies. Once an optimal signal-to-noise has been obtained from above, it may be of benefit to test several blocking and washing conditions to see if these will increase the S/N ratio.

Direct Assay Format

When coating proteins other than antibodies, a wider range of concentrations and possibly a variety of pHs will need to be tested. Fortunately, the 96-well format provides the ability to test varying coating concentrations in one direction while varying the antibody detection concentrations in the other direction. A recommended starting concentration of antigen would be in the range of 1 - 20µg/ml. Doubling dilutions

across the plate starting at 20µg/ml reaches 0.6µg/ml by the sixth column (likely to be nonsaturating). A different pH could be tested in columns 6 -12. Detection antibody could be started in row A at 500 ng/mL and reach 8 ng/mL by row G. Row H would receive no detection antibody and serve as the background control.

As above, once an optimal signal-to-noise ratio has been determined, additional variations in blocking can be tested to attempt further improvement.

Timing

The timing of an assay is typically a balance between keeping the procedure as short as possible and the need to reach equilibrium in order to achieve maximum sensitivity and specificity.

Typically the critical reactants are:

- primary antibodies
- secondary antibodies
- enzyme conjugates
- the material being coated
- the reference analyte

Blocking - Optimal blocking requires 1 - 2 hours to achieve, but in some assays 15 minutes may be sufficient. If the background is low, you are probably blocking sufficiently.

Antibody/Antigen - In a plate that is not stirred, it takes 3 - 4 hours to reach equilibrium, 1 hour is often sufficient. If positive results and sensitivity are in an acceptably readable range, the reagents are performing optimally.

Substrate - HRP substrates can be reacted for 10 - 20 minutes, beyond which more signal will not liekly be generated. it is not likely to produce additional signal. TMB produces a yellow color that should be stopped at 0.7 - 0.9 O.D. AP reactions can continue longer. As long as the background is not rising, the reaction you can proceed further.

Coating pH

Polyclonal Antibody	7 - 9
Monoclonal Antibody	7 - 9
Other	test several pHs near pI

Test Sample Effects

The accuracy of an immunoassay can be affected either positively or negatively by a variety of factors. Capture and detection antibodies that are cross-reactive are a prime example of a negative effect, but other sources are:

- interfering antibodies in the sample matrix
- antigens that are masked by binding to some matrix component
- matrix components that interfere with the enzyme activity or substrate conversion

"Recovery" Test

Often interferences can be detected if one has pure antigen (analyte) that can be used as a sample. A constant amount of sample antigen can be added into a dilution series of the matrix, and the detection of the sample measured quantitatively. If the amount of sample "recovered" is not what is expected, something in the matrix is interfering.

Changing lots or batches of critical reactants

Whenever a lot or a batch of a critical reactant needs to be changed, it is very important to only change one critical reactant at a time. Maintain enough of the old lot or batch to perform a test where the new lot can be compared for performance level to the old lot **on the same plate**. Keep all other reactants identical during this test.

Controls

Background

Proper controls are needed to account for any signal generated that is not due to the presence of the analyte under investigation.

There are a variety of reasons for background signal:

- nonspecific binding of analyte to the plastic
- presence of unexpected anti-Ig in the sample
- cross-reactivity of antibody to irrelevant antigens
- nonspecific binding of detection reagent to the plastic
- instability of the substrate

The following recommendations should help to pinpoint the source of background.

The best overall control is a sample of the matrix without the analyte. A satisfactory alternative is to add buffer to the control wells during the analyte-binding step.

If background does contribute significant signal, first test for high starting color in the substrate. Test for instability of the substrate by substituting buffer at the enzyme-conjugate addition step.

Test for nonspecific binding of enzyme conjugate by substituting buffer at the analyte addition step. Test for nonspecific binding of the analyte by leaving out the capture reagent when coating the plate. If it appears that significant background occurs at these steps, it is likely that insufficient blocking of the plate has occurred. Try blocking with either a higher concentration for a longer time or with a different blocking agent.

Cross-reaction of the detection antibody may be minimized by diluting the antibody with the matrix that does not contain any analyte.

Representative protocols

Direct ELISA

Apply Antigen

1. Add 100µl antigen diluted in coating solution to appropriate wells.
2. Incubate overnight at 4° C.
3. Empty plate and tap out residual liquid.

Block Plate

1. Add 300µl blocking solution to each well.
2. Incubate 15 minutes, empty plate and tap out residual liquid.

Add Antibody Conjugate Solution

1. Add 100µl antibody conjugate solution to each well.
2. Incubate 1 hour at room temperature (RT).
3. Empty plate, tap out residual liquid.

Wash Plate

1. Fill each well with wash solution.
2. Incubate 10 minutes RT.
3. Empty plate, tap out residual liquid.
4. Repeat 3-5 times.

React with Substrate

1. Dispense 100µl of substrate into each well.
2. After sufficient color development, add 100ul stop solution to each well.
3. Read plate with plate reader using appropriate filter.

Indirect ELISA

Apply Antigen

1. Add 100µl antigen diluted in coating solution to appropriate wells.
2. Incubate overnight at 4° C.
3. Empty plate and tap out residual liquid.

Block Plate

1. Add 300µl blocking solution to each well.
2. Incubate 15 minutes, empty plate and tap out residual liquid.

React with Primary Antibody

1. Add 100µl diluted primary antibody to each well.
2. Incubate 1-2 hours.
3. Empty plate, tap out residual liquid.

Wash Plate

1. Fill each well with wash solution.
2. Incubate 10 minutes RT.
3. Empty plate, tap out residual liquid.
4. Repeat 3-5 times.

Add Secondary Antibody Conjugate Solution

1. Add 100µl diluted secondary antibody conjugate to each well.
2. Incubate 1 hour RT.
3. Empty plate, tap out residual liquid and wash as above.

React with Substrate

1. Dispense 100µl substrate into each well.
2. After sufficient color development, add 100ul of stop solution to each well.
3. Read plate with plate reader.

Sandwich/Capture ELISA

Apply Capture Antibody

1. Add 100µl capture antibody diluted in coating solution to appropriate wells.
2. Incubate 1 hour RT.
3. Empty plate, tap out residual liquid.

Block Plate

1. Add 300µl blocking solution to each well.
2. Incubate 15 minutes, empty plate, tap out residual liquid.

React Sample Antigen

1. Add 100µl diluted antigen to each well.
2. Incubate 1 hour RT.
3. Empty plate, tap out residual liquid.

Wash Plate

1. Fill each well with wash solution.
2. Incubate 10 minutes RT.
3. Empty plate, tap out residual liquid.
4. Repeat 3-5 times.

Add Secondary Antibody Solution

1. Add 100µl diluted secondary antibody to each well.
2. Incubate 1 hour RT.
3. Empty plate, tap out residual liquid and wash as above.

React with Substrate

1. Dispense 100µl substrate into each well.
2. After sufficient color development, add 100µl stop solution into each well.
3. Read with plate reader.

Recommended filters

ABTS: 405-415nm

TMB: Unstopped 620-650nm; Stopped 450nm

*p*NPP: 405-415nm

BluePhos: 595-650nm

4. There are a lot but a few are critical.

Pipetting Tips

One of the most critical aspects of reproducible ELISA assays is to consistently deliver the same amount of liquid with a pipette.

Calibration

To insure proper calibration, pipette 10 replicates of water at the minimum volume of the pipette into a weigh boat. The CV of the replicates should be less than 2-3%. Repeat with 10 volumes at the maximum volume of the pipette. The CV should be less than 2-3%. If the CV is above 2-3% the pipette needs repair.

Pipetting Method

To reduce error due to surface tension of the liquid, the following method is recommended. Set the desired volume and pre-rinse the tip with liquid to be pipetted. Depress the plunger to the second stop. Draw in liquid slowly, allowing the plunger to return to the top. Let the liquid reach volumetric equilibrium. Dispense liquid to the first stop. Hold plunger at this stop until pipette is removed from the liquid. Slide the tip on the side of the well to remove any liquid held on the outside of the tip.

Pipetting Technique

- Maintain consistent speed while pipetting. Avoid sudden motions.
- Insure that the tip is firmly seated on the pipette.
- Change tips between each reagent.
- Use pipette within the range suggested by the manufacturer.
- Pre-rinse the tip with the reagent to be pipetted.
- If the reagent is viscous, pipette slowly and wait until the volume has reached equilibrium before removing the tip from the liquid.
- After drawing up liquid wipe tip with a lint-free tissue
- If an air bubble appears while pipetting, return liquid to the reservoir and re-pipette. If an air bubble continues to appear, replace tip.
- Use separate reservoirs for each reagent

Making Dilutions

It would seem tempting to make dilutions in a microwell plate using a multichannel pipettor by adding diluent to wells then adding reactant to a row, pipetting up and down a few times and transferring diluted reactant to the next row. There are several reasons not to do this. Pipetting up and down can cause foam, leading to inaccurate amounts being drawn up and an inaccurate dilution (sometimes only in one row of the plate). Secondly, in the time it takes to perform a mixing step either antigen-antibody interactions or adsorption to the plastic will take place, removing some of the reactant from the solution. Instead make all dilutions in low adsorbing test tubes (polypropylene or glass). Add each dilution to a reagent reservoir and add to the plate from the reservoir.

Avoid making large single step dilutions or dilutions which require measurement of a very low volume of reactant. If the dilution is more than 1/1,000, use two steps. For example: make a 1/100 dilution followed by a 1/10 dilution of the 1/100 diluted material. If the amount of reactant needed to make a dilution is 2 µl or less, prepare a larger amount than is needed in order to use a larger volume of reactant. This process will be more accurate.

Reminder:

To make 1.0 ml of a 1/100 dilution. Add 1µl of reagent to 99µl of diluent (not 1µl to 100µl). Better yet, add 5µl of reagent to 495µl of diluent.

Temperature

A critical factor for both the reactivity and the reproducibility of an ELISA is the temperature at which it is carried out. Most often reactions are carried out at room temperature, but the real source for error exists in not having all the wells come to the same temperature. As mentioned several times, polystyrene is a notoriously poor conductor of heat. The temperature of the reactants inside a well will reflect more closely the temperature which they had going into the well than the temperature of the room. If the temperature of the reactants was 4° C when put into the well, it can be 20 - 30 minutes before they come to room temperature. The step at which this is most critical is the substrate addition step. A "rule of thumb" for enzymes is that a 10° C change in

Insure that all components come to the temperature at which the reaction will be carried out before adding them to a plate.

temperature will result in a 2-fold change in activity. While the reaction mixture is not likely to have a 10° C well-to-well difference in temperature, even a 1° C temperature difference is noticeable.

Timing/Mixing

If reagents are not mixed while in the well, the rate of the reaction is diffusion-dependent. Even in an aqueous system of low viscosity it has been shown that it takes 3 - 4 hours for a binding step to approach equilibrium. If the medium is more viscous (e.g. serum), the rate will be even slower. Typically incubation times for the binding steps of 1 - 2 hours are recommended. Thus it is clearly not the case that they are at equilibrium. One drawback is the lowering of the sensitivity that occurs if binding equilibrium is not reached. However, a compromise between the level of sensitivity needed and the time constraints of completing the assay can usually be achieved. Since binding equilibrium is not likely to have been reached, the most important factor is to be consistent in the timing of the assay from day to day.

Mixing of the plate will speed up the time to reach the binding equilibrium. Vigorous mixing can reduce the required time from 3 - 4 hours to 1 - 2 hours. The key is to mix the assay the same from day to day.

The timing of the substrate conversion step is different. If one is using horseradish peroxidase (HRP), the rate of reaction will most likely slow after 20 minutes if the substrate is TMB. If ABTS is used, a longer incubation time may be possible. If one is using alkaline phosphatase (AP), reaction rates are slower than HRP, but will continue linearly for hours. HRP reaction rates slow due to irreversible substrate inhibition of the enzyme. One can follow the color change and stop the reaction when it reaches an intensity that is still within the range of readability of the plate reader (O.D. 1.0 - 2.0). When TMB is stopped, it changes color from blue to yellow. Color development increases in intensity approximately 2 - 3 fold and should be stopped when the O.D. reaches 0.7 - 0.9.

Critical Factors

Temperature

- Keep the temperature the same each time the assay is performed
- If the heat has been off over the weekend the lab bench may not be at "room temperature"
- Insure that all components are at the proper temperature before adding them to the assay
- If the assay is to be heated use a heating block rather than an air flow incubator

Timing/Mixing

- Determine the amount of time and/or mixing needed to attain the needed sensitivity and do it the same each time.

Standard Deviation and Coefficient of Variation

An issue that must be addressed in determining the value of the data generated is the variation of replicate determinations of the same concentration of analyte. An estimate of this is needed both to determine the precision of data points and the minimum amount of analyte that can be detected reproducibly above a determination of zero analyte.

In order to apply these estimates we must assume that the distribution of the values of replicate data points is normal or symmetrical (i.e. there is no skew) around the mean of the values. This may not always be the case and one should determine if the test conditions are causing a skew in the data. To simplify calculations the distribution is often assumed to be normal.

Note - If the values of the mean, the median and the mode of a set of data are identical or very similar, the data is normally distributed.

The standard deviation (SD) provides an estimate of the reproducibility of replicate data points and can provide confidence levels for assessing if one value is truly different from another. Whatever the measured value, a certain percentage of the values obtained are contained within the standard deviation. For instance, one SD on either side of the mean contains 68% of the values under the curve of that distribution. Approximately two SD (actually 1.96 SD) on either side of the mean contains 95% of all of the values and approximately three SD (actually 2.58 SD) contains 99% of all values. Thus if a value that is greater than 3SD different from the mean of a set of samples is obtained, one can be 99% confident that it is truly different from the first set of samples.

Mathematically, the SD is the square root of the sum of the variances squared divided by the number of samples minus one.

$$SD = \sqrt{(X-x_1)^2 + (X-x_2)^2 + (X-x_n)^2/n-1}$$

The Coefficient of Variation (CV) expresses the SD as a percentage of the mean.

$$\%CV = SD/mean \times 100$$

Limit of Detection

The lowest detectable analyte concentration that gives a response which has a statistically significant difference from the response of the zero analyte concentration is the detection limit. In order to have a confidence level of 95%, the means of the replicates of the zero analyte and the unknown concentration must differ by 2 SD and by 3 SD to have a 99% confidence level in the difference.

The factors that determine the ultimate sensitivity of a competitive assay are the antibody affinity constant and the experimental errors but not typically the detectability of the substrate. It has been calculated theoretically that with a $K = 10^{12}$ M^{-1} (an extraordinarily high constant for an antigen -antibody interaction) and a 1% CV for the response at zero dose, the lowest detection limit possible would be 10^{-14} M.

The factors limiting the sensitivity of a sandwich assay are the affinity of the antibody, the experimental error and the nonspecific binding of the labeled antibody, expressed as a percentage of the total antibody. It has been estimated that with a $K = 10^{12}$ M^{-1}, 1% CV of the response at zero dose, and a 1% nonspecific binding of the labeled antibody, the detection limit can be as low as 10^{-16} M . In addition, this can be enhanced further by using detection substrates with higher detectability.

Plotting the Data

The ELISA titration data that are generated when increasing concentrations of labeled analyte (or antibody) have been added are typically plotted either linear-linear; log-linear; log-log; or log-logit as illustrated in Figure 15.

Plots of Immunoassay Data

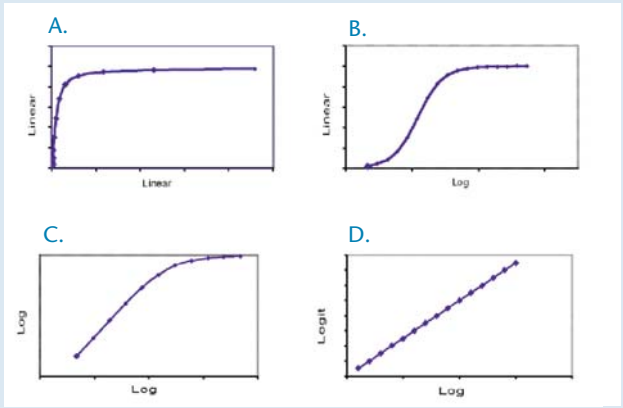


Figure 15.

A linear plot Figure 15A tends to compress the data points that are derived from the lowest concentrations where the interactions are least affected by steric hindrance, competition, or inhibition (the area of best precision). A log-linear (semilog) plot as illustrated in Figure 15B partially overcomes this effect and spreads the data into the typical sigmoid-shaped curve. This plot is often used to compare titration of two samples of labeled component. A more useful plot is illustrated in Figure 15C which shows a log-log plot of the data. In this case the region of receptor excess is completely linearized and ideally will have a slope of one. The linear region will allow a simple curve-fit by linear regression. The final plot (Figure 15D) is a log-logit plot which linearizes both the region of receptor excess and the region of saturation. A log-logit plot requires a highly precise estimate of the signal at saturation. An overestimate will not completely straighten the curve and an underestimate will result in an upward curving plot.

The most useful plot of the data is usually the log-log plot. It provides the most precise estimate of true values in the unsaturated region of the curve. It is easy to fit the data to a curve by linear regression. Deviations of the curve from the ideal are easy to discern and interpret.

Problem	Possible Cause	Solution
High Background	Insufficient Washing	• See washing procedure - page 14. • Add detergent to wash solution. • Increase number of washes. • Add 5 minute soak step between washes. • Add protein to the wash solution.
	Enzyme conjugate at too high a concentration	• Check dilution. Titrate if necessary.
	Insufficient Blocking	• Increase blocking protein concentration. • Try a different blocking protein. • Increase blocking time.
	Incubation times too long	• Reduce incubation time.
	Interfering substance in samples or standards.	• Run appropriate controls. • Perform "recovery assay" to determine masking effects.
	Contaminated buffers	• Make fresh buffers.
No Signal	Reagents added in incorrect order or incorrectly prepared	• Repeat assay. • Check calculations and make new buffers, standards, etc.
	Contamination of enzyme with inhibitor (azide for HRP or phosphate for AP)	• Use fresh reagents.
	Not enough reporter antibody used	• Increase concentration.
	Problems with the standard	• Check that standard was handled according to directions. • Use new sample.
	Capture antibody or analyte did not bind to plate	• Restandardize coating conditions. • Increase concentration of coating component. • Increase coating time. • Dilute antibody/analyte in phosphate buffer to insure that no other protein is present. • Change plate type to high binding. • Try covalent linkage plates.
	Buffers contaminated	• Make fresh buffers.

Problem	Possible Cause	Solution
Too much signal - plate uniformly reactive	Insufficient washing	• See washing procedure - page 14.
	Substrate solution changed color before use	• Use fresh substrate.
	Too much enzyme conjugate	• Check dilution. Titrate if necessary.
	Plate sealers or reagent reservoirs contaminated	• Use only fresh plate sealer and reservoirs.
	Buffers contaminated	• Make fresh.
Standard curve achieved but poor discrimination between points (low or flat slope)	Not enough enzyme conjugate	• Check dilution. Retitrate if necessary.
	Capture antibody did not bind well to plate	• Test different plate types. • Dilute capture antibody in phosphate buffer and insure that no other protein is present.
	Not enough detection antibody	• Check dilution. Retitrate if necessary.
	Plate not developed long enough	• Increase substrate incubation time.
	Incorrect procedure	• Go back to general protocol. Eliminate modifications.
	Improper calculation of standard curve dilutions	• Check calculations and make new standard curve.
Poor Duplicates	Insufficient washing	• See washing procdures on page 14. • If using an automatic plate washer, check that all ports are open and free of obstructions. • Add soak step - see page 15.
	Uneven plate coating due to procedural error or poor plate quality	• Dilute in phosphate buffer without additional protein. • Test coating buffers at different pH. • Check coating and blocking volumes, times and method of reagent addition. • Extend coating time to overnight. • Extend blocking time. • Use certified ELISA plates.
	Plate sealer reused or no plate sealer used	• Use new plates sealters each time.
	Buffers contaminated	• Make fresh buffers.

Problem	Possible Cause	Solution
Poor assay-to-assay reproducibility	Insufficient washing	• See washing procedure - page 14. • If using an automatic plate washer, check that all ports are open and free of obstruction.
	Variations in incubation temperature	• Bring all components to incubation temperature before adding to the wells. • Insure even heating of the plate-polystyrene is a poor conductor of heat.
	Variations in protocol	• Insure standard protocol is followed.
	Plate sealer reused	• Use fresh plate sealer for each step.
	Improper calculation of standard curve	• Recheck calculations. • Make new standard curve. • Use internal controls.
No signal where expected, but standard curve is fine	No analyte in sample or present at a concentration below the detection limit	• Recalibrate amount of sample to use.
	Sample matrix is masking detection	• Perform "recovery assay" to determine masking effects.
Samples reading above plate reader's ability to discriminate	Analyte concentration too high	• Dilute sample and rerun.
	Coating concentration too high	• Re-develop assay using the same concentration dilution factor for samples and coating solution.
Low reading across the entire plate	Incorrect wavelength on plate reader	• Check maximum absorbance range for the substrate being used. • Check filters.
	Insufficient development time	• Increase development time until background becomes detectable.
	Stored coated plates are inactive	• Coat new plates. • Treat with sucrose before drying.
	Coated component did not bind well to plate or at too low a concentration	• Re-titrate coating conditions.

Problem	Possible Cause	Solution
Edge Effects	Uneven temperature across plate	• Avoid incubating plates in areas where temperature fluctuations may occur. • Use plate sealers.

Microwell Plates

Corning - www.corning.com - click on “Products and Services” then click on “Life Sciences”. This site has a lot of information on plates and aspects of adsorption to solid surfaces.

Nunc - www.nalgenunc.com - click on “Products” then click on “Nunc Brand”. This site provides information on plate coating. In addition, search for "covalink" to find information on covalent attachment of molecules to microwells.

The above plates are also easily available through the following suppliers:
VWR - www.vwrsp.com search "microwell plates"
Fisher Scientific - www.fishersci.com search "microwell plates"

Antibodies

Linscott's directory at www.linscottsdirectory.com provides online information on sources of antibodies. Access is available for as little as $10.00.

Books - to order visit www.amazon.com

ELISA
Immunochemistry of Solid-Phase Immunoassay - ed. John E. Butler; CRC Press, 1991.

Immunoassay - ed. Eleftherios Diamandis and Theodore Christopoulos; Academic Press; 1996.

Antibodies
Using Antibodies - The Sequel to Antibodies, A Laboratory Manual - Ed Harlow and David Lane; Cold Spring Harbor Press; 1999.

Conjugating proteins
Bioconjugate Techniques - Greg Hermanson; Academic Press; 1996.

Web Sites

ELISA Assay - www.biology.arizona.edu. Under “Activites” click on “Immunology” or “ELISA”. This site illustrates an ELISA assay and describes what it measures and pitfalls in assays.

ELISA Assay - www.hhmi.org . Under "HHMI on the Web" click on "Biointeractive". On the “Biointeractive” page click on "Immunology" and "Virtual Labs". This site offers a virtual tour of an ELISA experiment.

The Centers for Disease Control (CDC) web site has a lot of information and many papers describing ELISA assasys. Go to the CDC home page www.cdc.gov and use the search engine with the term ELISA or EID (Emerging Infectious Diseases - a journal with open access to many papers featuring ELISA). There is also free downloadable Windows-based software for plotting ELISA data.

The following article is recommended as a starting point in developing an ELISA:
Quinn CP, et al. Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin-protective antigen. Emerg Infect Dis, Vol 8, No 10, Oct 2002, Available from: URL: http://www.cdc.gov/ncidod/EID/vol8no10/02-0380.htm

affinity The intrinsic attractiveness of one compound for another or the likelihood of staying together once having randomly come together. Between one binding site of an antibody and an epitope it is the three dimensional complementarity; hydrophobic; ionic, vanderWaals and hydrogen bonding forces that control the likelihood of staying together.

affinity purification A chromatographic purification step in which antibodies are passed over an antigen or epitope attached to a solid surface such as agarose. The antibodies specifically binding to the solid phase antigen can be recovered.

analyte The antigen, epitope or antibody to be measured by an ELISA.

antibody A four chain polypeptide that has affinity for a specific epitope. See - "The Use of Antibodies in Immunoassays"

antigen A molecule that can be recognized by antibodies. Large protein antigens often bear many epitopes; each one is usually distinct. Large carbohydrates, often made up of repeating identical subunits, will typically have repeating epitopes.

anti Ig Anti-immunoglobulin. An antibody that has affinity for an epitope found on an immunoglobulin molecule. For instance, goats immunized with mouse antibodies will make "goat anti mouse immunoglobulin". See - "The Use of Antibodies in Immunoassays"

ascities Fluid in the peritoneal cavity. To make ascities containing monoclonal antibody, mice are injected with an irritant to induce fluid and with hybridoma cells secreting a monoclonal antibody through the peritoneum.

avidity Properties of an antibody other than those defined as affinity that hold an antigen and antibody together and may be defined as the stability of the antibody-antigen complex. IgG and IgA have two binding sites per molecule and IgM has ten. An antigen such as a microbe may have multiple identical epitopes on its surface.

bound After a period of incubation some portion of the antigen, bearing reactive epitopes, will form a stable complex with one or more antibodies. Those antigens and antibodies which have formed a stable complex are referred to as the bound fraction.

capture antibody An antibody immobilized on a solid surface used to capture an epitope of interest from the test sample.

carrier A protein, to which a hapten can be attached, that will render the hapten capable of inducing an immune response.

coefficient of variation (CV) For normal (Gaussian) distributions, the coefficient of variation measures the relative scatter in data with respect to the mean. It is given as a percentage and is used to compare the consistency or variability of two more series. The higher the C V , the higher the variability, and lower the C. V., the higher the consistency of the data.

critical micelle concentration The concentration of a detergent above which it forms a micelle rather than being uniformly dispersed.

cross-reaction The observation that an antibody specific for one antigen may also react with a different antigen. This may occur when the two antigens share a common epitope or epitopes with similar three dimensional shapes so that the antibody can bind either one. Within populations of polyclonal antibodies cross-reactivity may also reflect the fact that one population reacts with one epitope while another population is specific for a different epitope.

ELISA Enzyme Linked Immunosorbant Assay. ELISA denotes a heterogeneous enzyme-based immunoassay in which one component is attached to a solid surface and enzyme-labeled antibody becomes bound through an epitope-antibody interaction. Unbound component is washed away before adding substrate to measure the amount of enzyme.

epitope A three-dimensional structure on the solvent-exposed portion of a molecule that interacts with an antibody-binding site.

equilibrium constant The equilibrium constant or K of an antigen - antibody interaction is the ratio of the on rate (k_a) to the off rate (k_d). The on rate is controlled by the concentration and mobility of the reactants (dependent on viscosity of the solution, size of the molecules, and whether the reactants are free in solution or restricted in mobility by adsorption to a solid surface). The off rate is controlled by the concentration and stability of the antigen - antibody complex (strength of the hydrophobic interactions, van der Waals forces, hydrogen bonding and ionic interactions that are holding the epitope in the antibody binding site). $K = k_a/k_d$ or K = [Ag-Ab]/ [Ag] [Ab].

F(ab) A fragment of an antibody molecule containing a light chain and a VH and a CH1. It has one epitope-binding site.

$F(ab')_2$ A F(ab) in which the CH1 has been extended to the hinge region and includes S-S bonds holding two F(ab) fragments together. It has two epitope binding sites.

Fc The C-terminal end of an antibody molecule containing the CH2 and CH3 domains. It does not contain any epitope-binding sites

free After a period of incubation some portion of the antigen bearing reactive epitopes will be in a stable complex with one or more antibodies. Those antigens which are NOT in a stable complex are referred to as the free fraction.

hapten A molecule, typically <1000 Dalton, that can be bound by an epitope-binding site, but cannot by itself induce an immune response. It is typically attached to a carrier to induce an immune response.

heterogeneous An assay in which the free component is washed away from the bound before making a reading.

homogeneous An assay in which the free and bound enzyme-conjugates do not need to be separated before making a reading. Typically some aspect of the binding step renders the bound enzyme-conjugate active while free enzyme-conjugate remains inactive.

hook effect A decrease in signal at high doses of analyte when more analyte is added to the assay. Also known as prozone.

hydrophillic Water-loving. Molecules that are hydrophillic go easily into solution in aqueous buffers.

hydrophobic Water-hating. Molecules that are hydrophobic are not easily dissolved in aqueous buffers and may require detergents or organic buffers to assist in dissolving them.

immunogen A molecule which, when injected into an animal, will induce an immune response.

monoclonal antibody Antibodies derived from one clone of cells. They will have the same binding site. Monoclonal antibodies are obtained by fusing an antibody-producing B cell with a cell line that has infinite ability to divide, then selecting a clone that produces the desired antibody. The resulting fused "hybridoma" cells can secrete the antibody derived from the B cell and have the ability to divide forever.

nitrocellulose (NC) A popular membrane used as the solid phase in Western blotting. It is a polymer of cellulose in which the --OH group has been modified to $--ONO_2$.

parallelism When the titration curve of the test and sample produce parallel lines.

pI Isoelectric point - The pH at which the net electric charge on a molecule is zero. On proteins the charge is due to $NH_2 \rightarrow NH_3^+$ and COOH COO^- .

polyclonal Each cell within a clone of B cells secretes identical antibody. When an antigen with multiple epitopes is injected, it is likely that several different clones of B cells will become activated to secrete. The resulting antiserum is referred to as polyclonal.

Protein A/G Protein A is a cell wall constituent of *Staphylococcus aureus*; while Protein G is derived from the cell wall of group G *Streptococcus*. Both have the ability to bind with high affinity to the Fc region of IgG of numerous mammalian species.

precision The agreement of replicate measurements. It is a measure of reproducibility but not of the accuracy of the results.

polyvinylidene fluoride (PVDF) A popular membrane used as the solid phase in Western blotting. It is a polymer of (CH2-$C[F_2]$)n . In contrast to nitrocellulose (NC), PVDF must be wetted by alcohol before use. It has a higher binding capacity than NC.

sensitivity The minimal detectable amount of an analyte that can be distinguished reproducibly from zero analyte. This is often referred to as the limit of detection.

standard curve A curve or straight line produced by mathematically fitting a curve to the data derived from a reference or known standard.

standard deviation The average amount by which data points deviate from the mean. It is the square root of the variance.

Western blot A solid phase immunoassay in which proteins are transferred from a polyacrlamide gel after electrophoresis to a membrane such as nitrocellulose or PVDF. After transfer detection of the protein bands can be accomplished using antibody-enzyme conjugates.

HRP ELISA Kits

AP ELISA Kits

Alkaline Phosphatase (AP) -labeled Antibodies

Horseradish Peroxidase (HRP) - labeled Antibodies

Biotin- labeled Antibodies

Labeled Streptavidin

Protein A

Substrates for ELISA

ELISA Peroxidase Chemiluminescent Substrates

BacTrace Antibodies to Bacteria

Assay Support Reagents and Accessories

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